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TERMINAL FLOWER2, an Arabidopsis Homolog of HETEROCHROMATIN PROTEIN1, Counteracts the Activation of *FLOWERING LOCUS T* by CONSTANS in the Vascular Tissues of Leaves to Regulate Flowering Time

Shinobu Takada[a,b,1] and Koji Goto[a,b,2]

[a] Research Institute for Biological Sciences, Okayama 716-1241, Japan
[b] Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Saitama 322-0012, Japan

The flowering time of plants is tightly regulated by both promotive and repressive factors. Molecular genetic studies using Arabidopsis have identified several epigenetic repressors that regulate flowering time. *TERMINAL FLOWER2* (*TFL2*), which encodes a homolog of HETEROCHROMATIN PROTEIN1, represses *FLOWERING LOCUS T* (*FT*) expression, which is induced by the activator CONSTANS (CO) in response to the long-day signal. Here, we show that *TFL2*, *CO*, and *FT* are expressed together in leaf vascular tissues and that *TFL2* represses *FT* expression continuously throughout development. Mutations in *TFL2* derepress *FT* expression within the vascular tissues of leaves, resulting in daylength-independent early flowering. TFL2 can reduce *FT* expression even when *CO* is overexpressed. However, *FT* expression reaches a level sufficient for floral induction even in the presence of *TFL2*, suggesting that TFL2 does not maintain *FT* in a silent state or inhibit it completely; rather, it counteracts the effect of CO on *FT* activation.

INTRODUCTION

The flowering of plants is regulated by many environmental stimuli and endogenous factors. The flowering of Arabidopsis is promoted by long days, gibberellins, and vernalization, but it can occur eventually even in the absence of environmental cues (autonomous promotion) (reviewed by Araki, 2001; Mouradov et al., 2002; Simpson and Dean, 2002). Recent findings suggest that the ambient temperature also affects the flowering of Arabidopsis (Blazquez et al., 2003).Thus, repressive factors as well as promotive factors are important in the regulation of flowering.

Molecular genetic screens of late and early flowering mutants of Arabidopsis have identified several genes involved in flowering and have found that external and internal floral promotion signals ultimately increase the expression levels of genes called floral pathway integrators, such as *LEAFY*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*)/*AGAMOUS-LIKE20*, and *FLOWERING LOCUS T* (*FT*) (Blazquez and Weigel, 2000; Lee et al., 2000; Samach et al., 2000). Among these flowering pathway integrator genes, *FT* seems to have great importance, because several flowering pathways, including the long-day, vernalization, autonomous promotion, and temperature-dependent pathways, are integrated into the regulation of *FT* expression. Moreover, loss-of-function *ft* mutations cause a severe late-flowering phenotype, and overexpression of *FT* causes an early-flowering phenotype that is independent of daylength and temperature (Kardailsky et al., 1999; Kobayashi et al., 1999; Blazquez et al., 2003).

In the long-day pathway, CONSTANS (CO), which is a B-box–type zinc finger protein that shares identity with GATA transcription factors, directly activates *FT* expression in response to long-day signals (Samach et al., 2000; Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002), and in the vernalization and autonomous pathways, *FLOWERING LOCUS C* (*FLC*) negatively regulates *FT* expression (Hepworth et al., 2002). Classic experiments suggest that the long-day pathway encompasses several processes that occur in different plant tissues: long-day signals are received by leaves, and signaling molecules generated in the leaves are transmitted to the shoot apical meristem (SAM) to induce flowering. Because of their low expression levels, however, the spatial expression patterns of *CO* and *FT* are poorly understood, making it difficult to predict the processes in which these genes are involved.

Recent findings from Arabidopsis research suggest that plants use a chromatin-mediated gene repression system to regulate flowering time. For example, VERNALIZATION2 (VRN2), a SU(Z)12-like Polycomb-group protein, is involved in the vernalization response through the stable silencing of *FLC* expression: *vrn2* mutations abolish the vernalization response and cause the derepression of *FLC* (Gendall et al., 2001). Mutations in two other Polycomb-group proteins, EMBRYONIC FLOWER2 [SU(Z)12-like] and FERTILIZATION-INDEPENDENT ENDOSPERM (EXTRA SEX COMB-like), also cause the precocious upregulation of several flowering genes, resulting in early-flowering phenotypes (Kinoshita et al., 2001; Yoshida et al., 2001; Moon et al.,

[1] Current address: Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630-0101, Japan.
[2] To whom correspondence should be addressed. E-mail kgoto@v004.vaio.ne.jp; fax 81-866-56-9454.
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2003). *TERMINAL FLOWER2* (*TFL2*), also called *LIKE HETEROCHROMATIN PROTEIN1*, encodes a protein homologous with an epigenetic repressor, HETEROCHROMATIN PROTEIN1 (HP1), which is involved in heterochromatin formation and the repression of some euchromatic genes in animals and fission yeast (Eissenberg and Elgin, 2000; Gaudin et al., 2001; Li et al., 2002; Kotake et al., 2003). Loss-of-function *tfl2* mutants show a daylength-independent early-flowering phenotype (Larsson et al., 1998; Kotake et al., 2003). Upregulation of *FT* in *tfl2* is the main cause of early flowering, because among known flowering genes only *FT* is upregulated in *tfl2* mutants and *ft* mutations completely suppress the early flowering of *tfl2* (Kotake et al., 2003).

It remains largely unknown how wild-type plants can overcome the epigenetic repression of flowering genes during floral induction. *TFL2* represents a good system in which to examine gene activation under such epigenetic repression, because *FT* is known to be activated directly by CO (Samach et al., 2000). Because TFL2 is expressed in various regions of plant tissues, in this study we initially characterized the spatial and temporal expression patterns of *CO* and *FT* in an attempt to understand their regulation. We found that both TFL2 and CO regulate *FT* expression in the vascular tissues of leaves, which suggests that CO and FT may have leaf-specific functions. With regard to the mechanisms of TFL2, we show that TFL2 can reduce, but cannot completely repress, the expression of *FT* by counteracting the activity of CO. Our results suggest that this counterbalance of TFL2 and CO activity on the expression of *FT* ensures the daylength-regulated flowering response of Arabidopsis.

RESULTS

Expression Patterns of *TFL2*, *CO*, and *FT* in Wild-Type Plants

To address whether TFL2 and CO regulate *FT* in the same tissue regions, we first analyzed the spatial expression patterns of these genes. As a result of their low expression, little is known about the expression domains of either *CO* or *FT*. Therefore, we generated transgenic plants using genomic fragments of *TFL2*, *CO*, and *FT*, which were sufficient to complement the respective *tfl2*, *co*, and *ft* mutants, coupled to the β-glucuronidase (GUS) reporter gene.

In *CO* gene (*gCO*):*GUS* transgenic plants, GUS was expressed in a *CO* genomic context: the start codon of an 8.4-kb genomic fragment spanning the *gCO*, which was sufficient to complement *co-101* (Table 1), was replaced with the GUS coding region (Figure 1A). For the detection of *TFL2*, we used *gTFL2*:*GUS* transgenic plants, in which a functional TFL2:GUS fusion protein was expressed in a *TFL2* genomic context (Figure 1A) (Kotake et al., 2003). To detect *FT* expression, an 8.9-kb region upstream of the *FT* start codon was fused to the GUS coding region (Figure 1A). An 11.8-kb genomic fragment (*gFT*) spanning the same 8.9-kb upstream sequence largely recovered the late flowering of *ft-101* (Table 1). Also, the expression of *FT* cDNA under the control of this 8.9-kb fragment was sufficient to rescue the late flowering of *ft-1* (Table 1), and this 8.9-kb promoter region seemed to contain regulatory sequences responsive to CO and TFL2 (see below). Moreover, both *gCO*:*co-101* and *pFT*:*FT*/*ft-1* transgenic plant lines delayed flowering time under short-day conditions (data not shown). Therefore, each construct contained the full regulatory sequence, indicating that the GUS expression pattern would mimic the expression pattern of endogenous mRNA, thereby facilitating highly sensitive whole-mount expression analysis, which is difficult to achieve by in situ hybridization.

In wild-type plants, the expression of *pFT*:*GUS* (i.e., an *FT* promoter fused to GUS) was seen first in the vascular tissues of cotyledons (Figure 1D) and was detected later in the vascular tissues of the apical part of the leaves (Figure 1P). GUS expression was not obvious in the primary veins or in the basal parts of the leaves, even in mature seedlings (Figures 1H and 1P). *pFT*:*GUS* expression was not continuous in vascular tissues and was interrupted (Figure 1P). *pFT*:*GUS* expression was not detected in leaf primordia, SAMs, hypocotyls, or roots (Figures 1D, 1H, and 2C). In inflorescences, *pFT*:*GUS* was expressed in the vascular tissues of inflorescence stems, pedicels, and floral organs but not in the inflorescence meristem (data not shown). Under short-day conditions, the expression pattern of *pFT*:*GUS* was similar to that under long-day conditions in 6-day-old seedlings (cf. Figure 1S with Figures 1D and 1L); however, after 8 days, *pFT*:*GUS* expression in true leaves diminished (Figure 1T). This photoperiod-dependent *pFT*:*GUS* expression also guarantees that this construct has full regulatory elements.

gTFL2:*GUS* was expressed in proliferating cells of leaves, SAMs, roots, and hypocotyls (Figures 1C, 1G, 1K, 1O, and 2B), as described previously (Kotake et al., 2003). *gTFL2*:*GUS* also was expressed in the vascular tissues of leaves and cotyledons (Figures 1C, 1K, and 1O).

gCO:*GUS* expression was detected in the vascular tissues of cotyledons and leaves (Figures 1B, 1F, 1J, and 1N). In leaves, GUS expression was detected first in the provascular tissues of leaf primordia and was detected later in the vascular tissues of whole leaves (Figures 1J, 1N, and 2A). *gCO*:*GUS* expression in roots depended on the line: 5 of 10 lines showed GUS expres-

Table 1. Complementation of *co*, *ft*, and *tfl2* Mutants

Plant	No. of Rosette Leaves	SE	*n*
Wild type (Col)	6.9	0.1	29
tfl2-2	4.9	0.2	9
gTFL2:GUS #17/tfl2-2	7	0.14	26
ft-101	43.7	1.93	6
gFT #1/ft-101	8.3	0.31	8
gFT #7/ft-101	8.5	0.29	4
ft-1 (Col)	36.1	1.49	7
pFT:FT #5/ft-1	6.5	0.27	21
pFT:FT #11/ft-1	7.8	0.2	20
co-101	16.7	0.87	7
gCO #7/co-101	7.8	0.31	20
gCO #18/co-101	7.3	0.35	29

Plants were grown on Murashige and Skoog (1962) plates containing 2% sucrose at a density of 30 plants/9-cm-diameter plate, except for *ft* and *co-101* mutants, which were grown at a density of 9 plants/plate. Kanamycin-resistant T2 progeny were used for analysis.

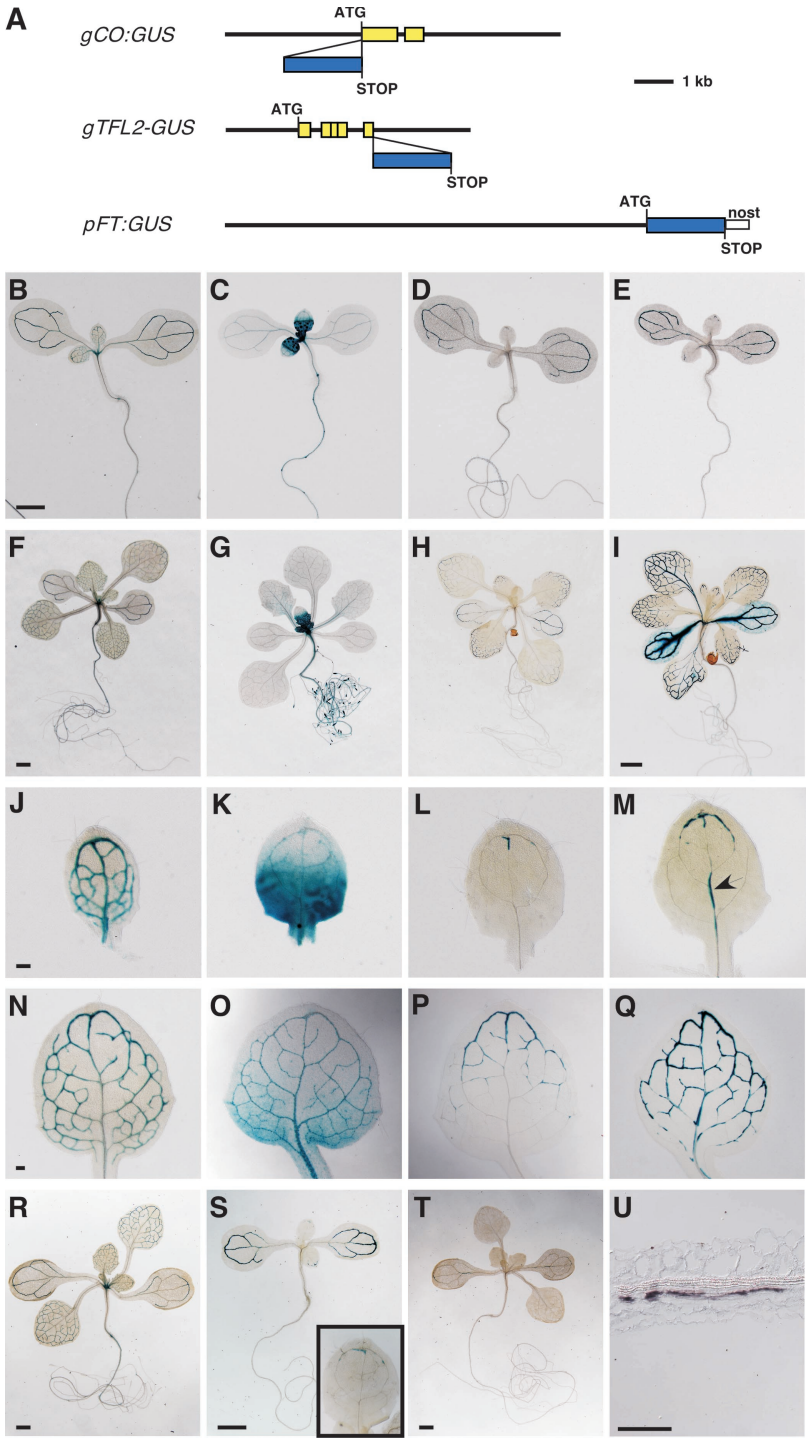

Figure 1. Whole-Mount Expression Analyses of *CO*, *FT*, and *TFL2*.

(A) Constructs used in expression analyses (see Methods). Yellow boxes indicate open reading frames. Blue boxes indicate the *uidA* open reading frame (*GUS* gene). ATG and STOP indicate what actually function, so that only *gTFL2*:*GUS* encodes a fusion protein. nost, the nopaline synthase terminator.

(B) to **(Q)** GUS expression patterns of *gCO*:*GUS* **([B]**, **[F]**, **[J]**, and **[N])**, *gTFL2*:*GUS* (**[C]**, **[G]**, **[K]**, and **[O]**), and *pFT*:*GUS* (**[D]**, **[H]**, **[L]**, and **[P]**) in ecotype Columbia (Col) and *pFT*:*GUS* in *tfl2-2* (**[E]**, **[I]**, **[M]**, and **[Q]**) in whole-mount staining of 6-day-old seedlings (**[B]** to **[E]**), 12-day-old seedlings (**[F]** to **[I]**), the first true leaves of 6-day-old seedlings (**[J]** to **[M]**), and the first true leaves of 8-day-old seedlings (**[N]** to **[Q]**).

(R) to **(T)** GUS expression in short-day conditions of 12-day-old *gCO*:*GUS* **(R)**, 6-day-old *pFT*:*GUS* **(S)**, and 12-day-old *pFT*:*GUS* **(T)** plants. The inset in **(S)** shows a higher magnification of the first true leaf.

(U) In situ hybridization against *FT* mRNA in the cotyledon of *tfl2-2* (longitudinal section).

The arrowhead indicates GUS expression in the primary vein in **(M)**. Bars = 1 mm in **(B)** for **(B)** to **(E),** 1 mm in **(F)** for **(F)** to **(H)**, 1 mm in **(I)**, 0.1 mm in **(J)** for **(J)** to **(M)**, 0.1 mm in **(N)** for **(N)** to **(Q)**, 1 mm in **(R)** to **(T)**, and 0.1 mm in **(U)**.

sion in root tips (data not shown). Like endogenous *CO*, *gCO*:*GUS* expression was not changed under short-day conditions (Figure 1R) (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002).

The expression patterns of both *gCO*:*GUS* and *gTFL2*:*GUS* in leaves were consistent with the results obtained by in situ hybridization (Simon et al., 1996; Kotake et al., 2003). *gCO*:*GUS* and *pFT*:*GUS* were expressed strongly in phloem within the vascular tissues (Figures 2E, 2G, and 2H). *gTFL2*:*GUS* was expressed in the epidermis, mesophyll, and phloem tissues of leaves (Figure 2F). These results suggest that, to regulate *FT* expression, TFL2 and CO must function in the phloem cells of leaves, because this is the only region in which all three of these genes are expressed.

tfl2 Causes an Increase in *pFT:GUS* Expression

To examine whether the expression of *pFT*:*GUS* was responsive to *TFL2*, lines expressing *pFT*:*GUS* in a *tfl2* background were crossed with the wild type, and total GUS expression was analyzed in F2 progeny. In *tfl2*, GUS activities were at least twice as high as those in the wild-type background on any day examined, showing that expression levels of *pFT*:*GUS* were repressed by TFL2 (Figure 3A). This result also suggests that the expression of *pFT*:*GUS* mimics endogenous *FT* expression.

pFT:*GUS* expression was detected earlier in *tfl2* than in the wild type (90% of first two true leaves in *tfl2* [$n = 30$] versus 27% in wild-type [$n = 18$] 6-day-old seedlings). Moreover, *pFT*:*GUS* was expressed ectopically in the primary veins of *tfl2* leaves (Figure 1M, arrowhead) (86.7% of first two true leaves in 6-day-old seedlings [$n = 30$]). This expression was never observed in the wild type (Figures 1L and 1P) (0% of first two true leaves in 6-day-old seedlings [$n = 18$]). In the *tfl2* background, the ectopic expression of *pFT*:*GUS* also was detected in hypocotyls (Figure 2D) but not in SAMs, young leaves, or roots (Figure 2D and data not shown). *FT* upregulation in *tfl2* hypocotyls suggested that *pFT*:*GUS* also is expressed in wild-type hypocotyls but at undetectable levels, consistent with previous studies showing that *FT* can be detected in wild-type hypocotyls using highly sensitive reverse transcriptase–mediated (RT) PCR techniques (Kardailsky et al., 1999; Kobayashi et al., 1999). In our in situ hybridization experiments, *FT* expression was not detected in the leaf vascular tissues in the wild type or *tfl2*, but it was detected in the vascular tissues of *tfl2* cotyledons, in which the strongest *pFT*:*GUS* expression was observed (Figures 1I and 1U). These results indicate that *FT* expression is upregulated with the same tissue specificity in *tfl2* and wild-type plants, suggesting that the upregulation of *FT* in the vascular tissues of leaves may be sufficient to cause early flowering.

co Affects the Upregulation of *FT* in *tfl2*

To assess the effect of *CO* on the upregulation of *FT* in a *tfl2* plant, we quantified *FT* expression in *co tfl2* double mutants (see Methods). Real-time quantitative RT-PCR analyses showed that the upregulation of *FT* in *tfl2* was largely suppressed by *co* in 6-day-old seedlings (Figure 3B), suggesting that CO and TFL2 antagonistically affect *FT* expression. In *co tfl2*, expression levels of *FT* were greater than in the wild type after 8 days (Figure 3C). *co tfl2* flowered as early as *tfl2* (Table 2, Figure 3E), suggesting that this upregulation of *FT* expression in *co tfl2*

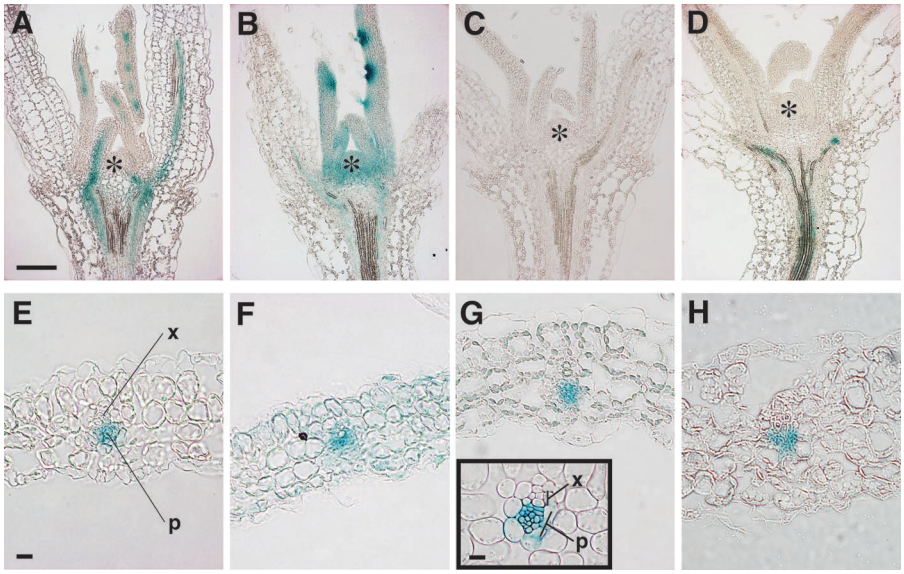

Figure 2. Histological Analyses of GUS Expression Patterns.

(A) to **(D)** Longitudinal sections through 8-day-old seedlings of *gCO*:*GUS* **(A)**, *gTFL2*:*GUS* **(B)**, and *pFT*:*GUS* **(C)** in Col and *pFT*:*GUS* in *tfl2-2* **(D)**.
(E) to **(H)** Transverse sections through leaves of *gCO*:*GUS* **(E)**, *gTFL2*:*GUS* **(F)**, and *pFT*:*GUS* **(G)** in Col and *pFT*:*GUS* in *tfl2-2* **(H)**.
Asterisks indicates the SAM. P, phloem; X, xylem. Bars = 0.1 mm in **(A)** for **(A)** to **(D)**, 10 μm in **(E)** for **(E)** to **(H)**, and 10 μm for the inset in **(G)**.

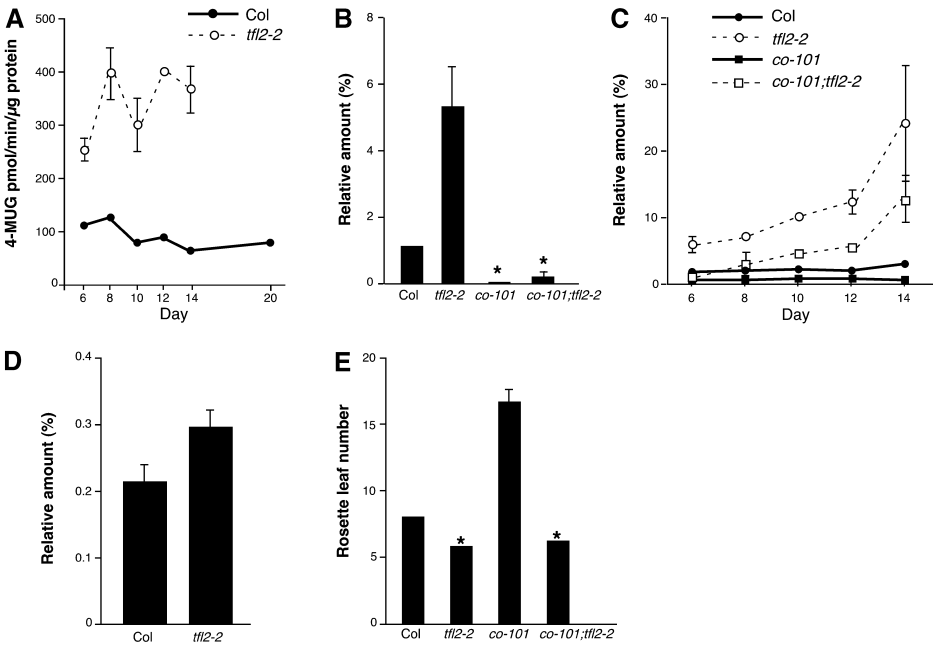

Figure 3. *tfl2* Affects Both the *CO*-Dependent and *CO*-Independent Expression of *FT*.

(A) Quantitative GUS expression analysis of *pFT*:*GUS* in Col (solid line) and *tfl2-2* (dashed line). GUS activity is shown as the mean ± SE of 4-methylumbelliferyl glucuronide·min^{-1}·μg^{-1} protein of three independent experiments.
(B) to **(D)** Real-time quantitative RT-PCR analysis of *FT* (**[B]** and **[C]**) and *CO* **(D)** expression. The same RNA extract was used for the experiments shown in **(B)** and **(D)**. These data are normalized to the amount of *ACT2* (set as 100%) and are means ± SE of three independent experiments.
(B) *FT* expression levels in 6-day-old Col, *tfl2-2*, *co-101*, and *co-101 tfl2-2* seedlings.
(C) *FT* expression over time in Col, *tfl2-2*, *co-101*, and *co-101 tfl2-2* seedlings.
(D) *CO* expression levels in 6-day-old Col and *tfl2-2* seedlings.
(E) Flowering time measured as the mean number (±SE) of rosette leaves at flowering in Col, *co-101*, *tfl2-2*, and *co-101 tfl2-2* (n = 27, 7, 27, and 9, respectively). All plants were grown under long-day conditions. Asterisks indicate data that do not show statistically significant differences.

after 8 days is sufficient for early flowering. This result also supports the notion that the early flowering of *tfl2* is not attributable to the upregulation of *CO* (Kotake et al., 2003).

In the *co* mutant background, *pFT*:*GUS* expression was not detected in 69.6% of leaves and was weakly detected in 30.4% of leaves in 12-day-old seedlings (n = 23) (Figures 4B and 4E). In *co tfl2*, however, *pFT*:*GUS* expression was detected in the basal parts of leaves (78.2% of leaves in 12-day-old seedlings [n = 23]) (Figures 4C and 4F). These results suggest that the expression of *FT* in the apical parts of leaves requires the activity of CO and that CO-independent *FT* upregulation in *tfl2* occurs mainly in the basal parts of leaves.

In 6-day-old seedlings, *FT* expression depends largely on CO, and the expression levels of *CO* were similar in *tfl2* and wild-type plants (Figure 3D); however, the *CO*-dependent expression of *FT* was approximately five times greater in *tfl2* than in the wild type (Figure 3B). These findings, together with the fact that *FT* was upregulated in the *CO*-expressing tissues of *tfl2*, indicate that TFL2 may alleviate the effect of CO on *FT* activation.

Table 2. Flowering Time of Mutant and Transgenic Lines

Plant	No. of Rosette Leaves	SE	*n*
Wild type (Col)	8.1	0.15	27
tfl2-2	5.9	0.15	27
co-101	16.7	0.87	7
co-101 tfl2-2	6.3	0.17	9
35S:CO	4.6	0.14	20
35S:CO tfl2-2[a]	3.5	0.33	8
35S:TFL2 #16	6.9	0.4	8
35S:TFL2 #26	7.5	0.37	10
35S:TFL2 #16/+ 35S:CO/+	5	0	9
35S:TFL2 #26/+ 35S:CO/+	5	0.27	8
35S:CO/+	5	0	9

Plants were grown on Murashige and Skoog (1962) plates containing 2% sucrose at a density of 9 plants/9-cm-diameter plate. F1 plants were grown on plates containing kanamycin (50 μg/mL).
[a] Includes both *35S:CO/+ tfl2-2* and *35S:CO tfl2-2*.

tfl2 Enhances the Early-Flowering Phenotype and Upregulation of *FT* in *35S:CO* Plants

We next examined whether TFL2 could counteract the activity of CO in a *CO* overexpressor line, *35S:CO*, which shows a day-length-independent early-flowering phenotype (Putterill et al., 1995; Simon et al., 1996). Quantitative RT-PCR analysis showed that expression levels of *FT* were approximately twice

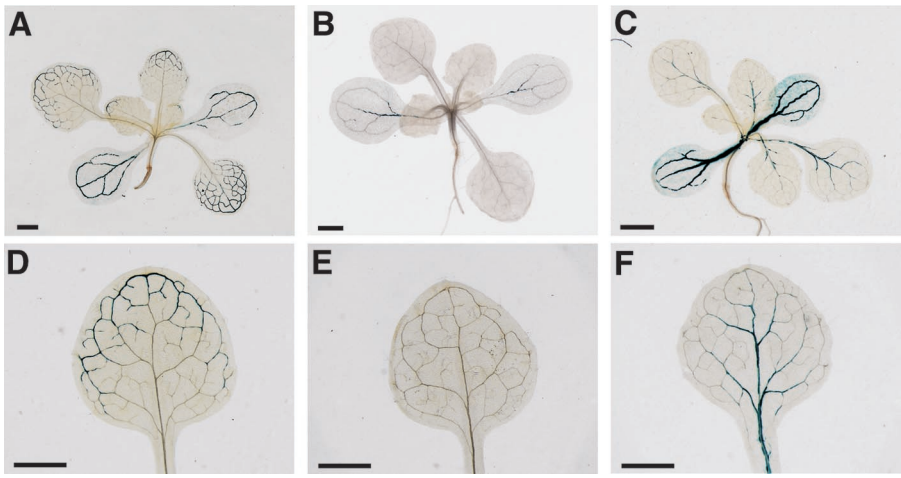

Figure 4. *pFT*:*GUS* Expression Requires CO Activity.

pFT:*GUS* expression is shown in 12-day-old seedlings of wild-type Col **(A)**, *co-101* **(B)**, and *co-101 tfl2-2* **(C)** (roots were cut off for genotyping) and in leaves of wild-type Col **(D)**, *co-101* **(E)**, and *co-101 tfl2-2* **(F)**. Bars = 1 mm.

as high in *35S*:*CO tfl2* plants as in *35S*:*CO* plants (Figure 5A). Moreover, *35S*:*CO tfl2* flowered earlier than *35S*:*CO* (Figure 5B), reflecting the higher expression levels of *FT*. These findings demonstrate that *TFL2* can reduce the expression of *FT* even in the CO overexpressor line *35S*:*CO*.

To examine the differences in the expression patterns of *FT* between *35S*:*CO* and *35S*:*CO tfl2*, the *pFT*:*GUS* transgene was introgressed into the *35S*:*CO* and *35S*:*CO tfl2* backgrounds (see Methods). *pFT*:*GUS* was expressed ectopically in hypocotyls, roots, and inflorescences of both *35S*:*CO* and *35S*:*CO tfl2* (Figures 5C and 5D). GUS expression also was observed in the primary veins of leaves in both the *35S*:*CO* (91.7% of the first two true leaves in 8-day-old seedlings [$n = 36$]) and *35S*:*CO tfl2* (95.7% of the first two true leaves in 8-day-old seedlings [$n = 47$]) backgrounds. We found no significant differences in the expression patterns of *FT* within tissues between *35S*:*CO* and *35S*:*CO tfl2* (Figures 5C and 5D), indicating that in *35S*:*CO*, the expression patterns of *FT* are determined mainly by *CO* and are independent of the presence of *TFL2*.

In contrast to the loss of *TFL2* expression, overexpression of *TFL2* (*35S*:*TFL2*) showed little effect on flowering time and the expression levels of *FT* in both the wild-type and *35S*:*CO* backgrounds (Table 2 and data not shown), suggesting that an increase in *TFL2* expression alone does not affect flowering times.

DISCUSSION

Here, we have documented the spatial and temporal expression patterns of *FT*, which were poorly known previously. Our results suggest that *FT* expression in these tissues is sufficient for daylength-dependent flowering and is regulated by the activator CO and the repressor TFL2. Although TFL2 is homologous with HP1, TFL2 is not involved in an on/off switch of *FT*, because *TFL2* does not determine the timing or the spatial pattern of *FT* expression. Rather, TFL2 counteracts the activity of activators such as CO on *FT* expression to ensure daylength-dependent flowering, because only a small change in the normal expression of *FT* is sufficient to induce flowering that is independent of daylength.

TFL2 Counteracts the Activation of the Transcription of *FT*

Three lines of evidence suggest that *TFL2* counteracts the activation of *FT* by CO: (1) *CO*-dependent *FT* expression is enhanced in *tfl2*; (2) *FT* expression is upregulated in the *CO*-expressing tissues of *tfl2*; and (3) overexpression of *CO* induces higher levels of *FT* in the absence of *TFL2* than in its presence. However, the fact that *FT* was still upregulated in *co tfl2* (in 8-day-old seedlings and in the later stages) implies that activators other than CO also are involved in the activation of *FT*. These factors have been proposed in previous studies, because *FT* expression increases gradually in later seedling development even in *co* mutants (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000). In the *tfl2* background, *FT* is upregulated in the presence or absence of CO. A simple model to explain this CO-independent *FT* upregulation is that TFL2 directly represses *FT* expression.

How does TFL2 reduce the effect of transcriptional activators in the cells? TFL2 is homologous with HP1, which represses gene expression in heterochromatin by binding directly to histone H3 tails (methylated K9) to form higher order chromatin structures (Lachner et al., 2001); therefore, TFL2 may localize to the chromatin of *FT* to control transcription. In contrast to the proposed function of HP1 in heterochromatin formation in fly and mammals, however, TFL2 cannot completely inhibit but can only counteract the activities of transcription factors. This "offsetting" of the activity of the activator, as opposed to the stable repression observed (e.g., in the repression of *FLC* by the Polycomb-group protein VRN2), suggests a novel role of HP1-type repressors. In other words, TFL2 may constitute a component of the dynamic structure of silent chromatin that

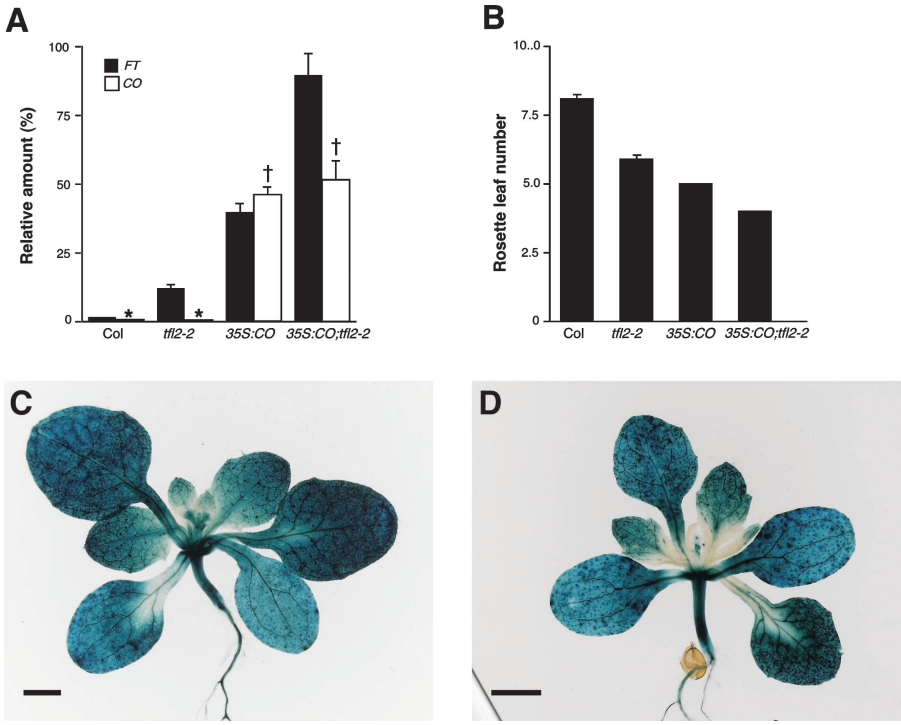

Figure 5. Roles of *TFL2* during *FT* Repression in the *CO* Overexpressor.

(A) Real-time quantitative RT-PCR analysis of *FT* (closed bars) and *CO* (open bars) expression in 12-day-old seedlings of Col, *tfl2-2*, *35S*:*CO*, and *35S*:*CO tfl2-2*. The data are normalized to the amount of *ACT2* (set as 100%) and are means ± SE of three independent experiments. There was no statistically significant difference among genotypes marked with the same symbols.

(B) Flowering time measured as the mean number (±SE) of rosette leaves at flowering in Col, *tfl2-2*, *35S*:*CO*, and *35S*:*CO tfl2-2* (n = 27, 27, 43, and 22, respectively).

(C) and **(D)** Whole-mount analysis of *pFT*:*GUS* expression in *35S*:*CO* **(C)** and *35S*:*CO tfl2-2* **(D)**. Bars = 1 mm.

does not completely inhibit but only reduces the accessibility of transcription factors, as proposed recently in the "site-exposure model" (Ahmad and Henikoff, 2002).

TFL2 Is a Regulator of the Expression Levels of *FT*

Our results suggest that TFL2 does not determine the timing of floral transition in wild-type Arabidopsis, because the expression of *TFL2* was not downregulated before flowering and *35S*:*TFL2* did not show late flowering (Table 1). Moreover, the expression level of *TFL2* was not altered between long-day and short-day conditions, and *TFL2* expression did not show circadian oscillations, suggesting that TFL2 activity itself does not change in response to daylength (our unpublished data). Rather, TFL2 functions to maintain a low level of *FT* both before and after the floral transition, because the expression levels of *FT* in the wild type never reached those in *tfl2* even after the floral transition (Figure 3C). This mechanism of regulation differs from that used by *FLC*, whose expression must be diminished to induce flowering (Sheldon et al., 2000). Moreover, overexpression of *FLC* causes a strong repression of both *FT* and *SOC1* even in the *35S*:*CO* background (Hepworth et al., 2002), whereas *TFL2* can only reduce the expression level of *FT* and has little role in determining the tissues that express *FT* in the *35S*:*CO* background. Therefore, unlike other HP1 proteins, TFL2 seems to generally reduce gene expression levels and does not distinguish between active and silent states.

What is the biological relevance of the TFL2-mediated repression of *FT*? One interpretation is that only a small amount of *FT* is needed to induce flowering; therefore any enhanced response to CO will cause an accumulation of *FT* before floral induction and will disrupt the long-day-regulated flowering of plants. Thus, TFL2 may be expressed continuously to offset any excess activity of CO. In addition, even *35S*:*CO tfl2* could not induce *pFT*:*GUS* expression in young leaves, root tips, or 8-day-old SAMs (data not shown), suggesting that the existence of other repressors or the absence of coactivators may limit the activity of CO on *FT* expression in these tissues. EARLY BOLTING IN SHORT DAYS (EBS), a putative chromatin-remodeling factor that specifically represses *FT* expression (Gomez-Mena et al., 2001; Pineiro et al., 2003), is a candidate for another repressor that modulates the CO responsiveness of *FT*. In our yeast two-hybrid screening, however, TFL2 did not interact with EBS (our unpublished data), suggesting that TFL2 and EBS may form a different protein complex and may function independently. The existence of at least two repressors suggests that the strict regulation of *FT* expression levels is critical for the correct flowering time during plant development.

TFL2 and CO Regulate *FT* Expression in the Vascular Tissues of Leaves

Classic experiments have suggested that inductive photoperiods are recognized by leaves and that signaling molecules produced in the leaves are transmitted into SAMs to induce flowering (reviewed by Colasanti and Sundaresan, 2000). In addition, the expression of the maize *INDETERMINATE* gene only in leaves has been shown to be sufficient for floral promotion (Colasanti et al., 1998), indicating the importance of the expression of flowering genes in leaves. The expression of *FT* in leaves suggests that *FT* may be involved in a floral promotion signaling pathway in leaves; *FT* activation by CO may cause the generation of unidentified signal molecules in the vascular tissues of leaves, and these signals (or FT itself) may be transmitted via vascular tissues to SAMs to induce flowering. In potatoes, the Arabidopsis *CO* gene was found to act non-cell-autonomously to delay tuber formation, which is induced in short days (Martinez-Garcia et al., 2002). Our results show that in Arabidopsis, CO itself seems not to act non-cell-autonomously, because CO activates *FT* expression only in *CO*-expressing tissues, raising the possibility that *FT* and/or its downstream gene(s) may act non-cell-autonomously to induce flowering.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia (Col) was used as the wild type. We searched T-DNA insertion lines provided by the Torrey Mesa Research Institute and found *co* and *ft* mutant alleles in the Col background, named *co-101* and *ft-101*, respectively. *ft-1* was introgressed into Col and *tfl2-2* is a null allele, as described previously (Kotake et al., 2003). *35S*:*CO* transgenic plants in the Col background were a gift from G. Coupland (Max Planck Institute for Plant Breeding, Cologne, Germany).

To obtain *co-101 tfl2-2* double mutants (*CO* and *TFL2* loci are only ~2 centimorgan apart), *co-101* was crossed with *tfl2-2*. In F2 progeny, *co-101*/+ *tfl2-2* seedlings were identified on the basis of BASTA resistance and the *tfl2* phenotype, and *co-101 tfl2-2* was obtained in the next generation. Genotypes were confirmed by PCR, and F4 plants were used for analysis.

To obtain *35S*:*CO tfl2-2* plants, *35S*:*CO* was crossed with *tfl2-2*. *35S*:*CO tfl2-2* was identified by screening kanamycin-resistant F2 seedlings for a curled-leaf phenotype, and homozygosity for *tfl2-2* was confirmed by PCR. Because of the low fertility of *35S*:*CO tfl2-2*, F2 plants were used for flowering-time and expression analyses.

For flowering-time and expression analyses, plants were grown on Murashige and Skoog (1962) agar plates with 2% sucrose in long-day conditions (16 h of light/8 h of dark) under white fluorescent lights (~50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or short-day conditions (10 h of light/14 h of dark; ~50 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Sown seeds were kept for 2 days at 4°C and then moved to 22°C, which was defined as day 0 after sowing.

Plasmid Construction and Transgenic Plants

gCO:co-101

An 8420-bp *CO* genomic fragment containing a region encompassing 3576 bp upstream of the start codon to 3489 bp downstream of the stop codon was excised from BAC clone F14F8, cloned into the pCGN1547 plant transformation vector (Calgene, Davis, CA), and transformed into *co-101*. Among T1 plants, 13 of 20 lines flowered earlier than *co-101* and produced no more than 10 leaves. Two T2 lines were used to count the number of rosette leaves at flowering.

gCO:GUS

To make *gCO*:*GUS*, the start codon of the 8420-bp *CO* genomic fragment was replaced with *uidA* cDNA. Whole-mount GUS expression analysis revealed that 10 of 11 T2 lines showed basically the same expression pattern. Four strong lines, carrying a single locus insertion of the transgene, were sectioned. T2 seedlings were used for analysis.

gFT:ft-101

An 11,781-bp *FT* genomic fragment extending from 8,902 bp upstream of the start codon to 699 bp downstream of the stop codon was excised from BAC clone F5I14, cloned into the pCGN1547 vector, and used to transform *ft-101*. Two independent T2 lines were obtained and used for flowering-time analysis.

pFT:FT/ft-1

Introns were removed from the 11.8-kb *FT* genomic fragment by reference to the *FT* cDNA, and the 3′ downstream sequence was replaced with the nopaline synthase terminator. Thus, this construct contained 8.9 kb of 5′ upstream sequence and the coding region of the *FT* gene. In T1 plants, 12 of 20 independent lines flowered earlier than *ft-1* and produced no more than 10 leaves. Two T2 lines were used to count the number of rosette leaves at flowering.

pFT:GUS

An 8.9-kb region upstream of the start codon of *FT* (the same region as in *pFT*:*FT*) was fused to the GUS coding sequence followed by the nopaline synthase terminator in the pCGN1547 vector. This construct was used to transform Col and *tfl2-2*. In the Col background, 9 of 15 T2 lines showed a similar expression pattern. In the *tfl2-2* mutant background, 8 of 8 T2 lines showed a similar expression pattern. In general, GUS staining was stronger in *tfl2-2* than in Col, and GUS expression in the primary veins of leaves was rarely observed in Col background, whereas all eight lines in the *tfl2-2* background showed GUS expression in the primary veins. Two strong lines in the *tfl2-2* background were sectioned, and four strong lines in the Col background were sectioned. For histological GUS staining, T2 seedlings were used. Two lines in the *tfl2-2* background, carrying a single locus insertion of the transgene, were used for crossing with Col. GUS activity was compared between *pFT*:*GUS*/Col and *pFT*:*GUS*/*tfl2-2* segregated in F2 progeny. To examine the expression pattern of *pFT*:*GUS* in the *35S*:*CO* and *35S*:*CO tfl2-2* backgrounds, a *pFT*:*GUS* line in the *tfl2-2* background was crossed with *35S*:*CO*, and the expression patterns of GUS were analyzed in F2 progeny. To obtain *pFT*:*GUS co-101* and *pFT*:*GUS co-101 tfl2-2*, a *pFT*:*GUS* line in the Col background was crossed with *co-101* and *co-101 tfl2-2*, respectively, and kanamycin-resistant F2 seedlings were genotyped by PCR.

35S:TFL2

To obtain *35S*:*TFL2*, *TFL2* cDNA was inserted between the 35S promoter of *Cauliflower mosaic virus* and the nopaline synthase terminator in the pCGN1547 vector and used to transform Col. Two independent T3 homozygous lines, showing overexpression of *TFL2* (data not shown), were used for analysis. To obtain *35S*:*TFL2*/+ *35S*:*CO*/+, *35S*:*TFL2*

lines were crossed with *35S*:*CO*, and F1 plants were used for flowering-time analysis.

Expression Analyses

Samples were collected at dusk, when the expression of *FT* is the highest (Suarez-Lopez et al., 2001; Kotake et al., 2003).

Real-Time Quantitative PCR

Real-time quantitative PCR using TaqMan probes was performed as described previously (Kotake et al., 2003) with the following modifications. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA) and was treated with RNase-free DNase (Qiagen) according to the manufacturer's instructions. Two micrograms of total RNA was reverse-transcribed using Omniscript reverse transcriptase (Qiagen). cDNA was resuspended in 200 μL of water, and 2.5-μL aliquots were analyzed.

Histological Analysis of GUS Staining

GUS staining was performed as described previously (Honma and Goto, 2000). Samples were embedded in paraffin and sectioned at a thickness of 8 μm with a microtome. For the section shown in the inset of Figure 2G, samples were embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) and sectioned at a thickness of 5 μm.

GUS Activity Measurement

For quantitative GUS measurements, we used an assay based on the substrate 4-methylumbelliferyl glucuronide, as described (Jefferson et al., 1987). The protein concentrations of samples were determined using a bicinchoninic acid assay kit (Pierce).

In Situ Hybridization

In situ hybridization was performed as described by Kotake et al. (2003). The full-length *FT* cDNA clone kindly provided by T. Araki (Kyoto University) was used as template for the *FT* probe.

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact K. Goto, kgoto@v004.vaio.ne.jp.

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